Evidence of local oxidative stress in canine senile cataract

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SUMMARY

Constant exposure to solar radiation predisposes the eye to intense burden of reactive oxygen species (ROS), making it highly vulnerable to oxidative damage. The aim of the present study was to evaluate some oxidative stress parameters and related trace minerals in the aqueous humor and lens of dogs affected by senile cataract. Six dogs with age-related cataract (test group) and 6 dogs with healthy eyes (control group) were included in the study. The concentrations of some products of lipid and protein peroxidation, as well as antioxidant capacity and related trace minerals were measured in the lens and aqueous humour and compared between the two groups. The results showed a significant elevation of malondialdehyde (MDA), protein carbonyls (PCO), advanced oxidation protein products (AOPP), as well as reduction of glutathione (GSH) and iron (Fe) levels in aqueous humor of eyes with cataract (4.45 ± 3.76 µmol/l; 27.7 ± 19.7 µmol/l; 3.07 ± 1.74 µmol/l; 152 ± 190 µmol/l; 28.3 ± 10.8 µmol/l, respectively) in comparison with non-affected eyes (1.98 ± 0.71 µmol/l; 4.06 ± 3.21 µmol/l; 1.04 ± 0.45 µmol/l; 399 ± 231 µmol/l; 53 ± 19.2 µmol/l, respectively); MDA and Fe but not PCO and AOPP were also increased in cataractous lenses (0.85 ± 0.50 µmol/g; 0.53 ± 0.03 µmol/g; 1.51 ± 2.13 µmol/g; 4.34 ± 2.55 µmol/g, respectively) while GSH and ferric reducing ability (FRAL) decreased significantly (20.8 ± 8.9 mol/g; 3.34 ± 2.32 mol/g, respectively) compared to the healthy ones (0.05 ± 0.03 µmol/g; 0.46 ± 0.05 µmol/g; 2.21 ± 1.94 µmol/g; 3.39 ± 2.86 µmol/g; 41.3 ± 12.1 mol/g; 7.02 ± 1.60 mol/g, respectively). Our study confirmed the occurrence of lipid and protein peroxidation in eyes of dogs with senile cataract. Moreover, local antioxidant defense seemed to be insufficient to overcome excessive ROS production.

Keywords: cataract, dogs, oxidative stress, trace minerals

Reference

Cataract formation is one of the leading causes of vision loss worldwide, accounting for 33.4% of all causes of blindness both in humans [23] and dogs [4]. Lifetime cataract incidence in the large total studied population of over 100,000 dogs without congenital and juvenile hereditary cataracts was 9.07±5.69% [39]. Cataract incidence between the 7th and 10th year of life was 14.10±8.96%. Some sources only reported cataract prevalence in dogs above 7 years of age to exclude the incidence of hereditary cataracts [15].

Current medical therapy of cataract gives unsatisfying results, whereas surgical removal by phacoemulsification may lead to intra- and postoperative complications such as retinal detachments, glaucoma, vitreous body leakage, uveitis, etc. In this context, the quest for alternatives to prevent and treat canine cataracts seems to be worthwhile. The prerequisite is first to better know the biochemical changes associated with the development of lens opacities in dogs.

Age-related cataract is characterized by lens protein aggregation and fiber cell plasma membrane damage, which causes clouding of the lens, light scattering, and vision loss. It is also associated with an intralenticular presence of large amounts of reactive oxygen species (ROS) in rats and mice [35, 41]. ROS-induced damage in the lens cell may comprise oxidation of proteins, DNA damage, and/or lipid peroxidation, all of which have been implicated.
in cataractogenesis. Oxidative stress and DNA damage contribute to the pathogenesis of age-related cataract in humans [40]. These events also may occur in age-related cataracts in dogs.

The majority of the surveys were accomplished on human cell or tissue cultures or on experimentally induced cataract in mice and rats. Data about oxidative stress in lens and aqueous humor from spontaneous canine cataract are scarce. Moreover, human lenses are quite different in many important ways from their animal counterpart [38] with regard to differences in lens protein and lipid content, UV filters, antioxidant enzyme activities, compaction of the lens, synthesis of ascorbate etc. Therefore, it is relevant that species specific studies are made.

The aim of the present study was to investigate the levels of some oxidative stress parameters and related trace minerals in the aqueous humour and lens of dogs affected by senile cataract.

**Materials and methods**

**ANIMALS**

Six dogs from both sexes with mean age of 11.6 years affected by age-related mature cataracts in one or both eyes, which underwent cataract surgery using phacoemulsification (Premiere™ model DP1402-200, Storz Instrument Company, St. Louis, MO, USA), were included in the study and formed the test group. Animals with toxic, diabetic, infectious, or traumatic cataracts, determined on the basis of detailed clinical, laboratory and ophthalmological examination, were excluded from the trial. Those aged less than 7 years were also excluded to safeguard against hereditary cataracts.

Another group of animals over 7 years of age, dead or euthanized for unrelated causes served as a control group. Their healthy eyes, determined by ophthalmological examination, were immediately processed for sampling and storage.

**SAMPLE COLLECTION**

Just before surgery or just after death, 0.5 mL of aqueous humour was aspirated through paracentesis of anterior chamber using 25G needles and immediately stored at -70°C for further examination.

The lenses from healthy eyes were taken out, washed in cold distilled water, and weighed. Each lens was first fragmented by phacoemulsification and homogenized in 10-fold volume of iced potassium phosphate buffer (pH 7.2) supplemented with potassium chloride using a portable homogenizer (Tissue-Tearor 985370-14, BioSpec, USA) at 35,000 rpm for 30 s. The mixture was centrifuged at 12,000 rpm (25 min), the supernatant was aliquoted and stored at -70°C until biochemical analysis.

The fragments of the cataractous lenses obtained during phacoemulsification were collected in 150 mL plastic containers. The large fragments were centrifuged, washed in cold distilled water, weighed, and homogenized as described above [32]. The supernatant was divided in portions and stored at -70°C.

**PARAMETERS AND ASSAY METHODS**

The total protein in the supernatant was estimated using the biuret test.

**Oxidative stress parameters**

**Malondialdehyde (MDA)** – the method measuring the amount of thiobarbituric acid (TBA) reactivity by the amount of malondialdehyde (MDA) formed during acid hydrolysis of the lipid peroxide compounds was used [2, 31]. In brief, the aqueous humor or lens homogenate sample (0.3 mL) was mixed with 3 mL of 1% orthophosphoric acid, 1 mL of 0.6% thiobarbituric acid aqueous solution and 0.1 mL freshly prepared ferrous sulfate solution (28 mg FeSO₄·7H₂O in 10 mL water). After thorough homogenisation, the mixture is heated on a boiling water bath for one hour. After cooling, 4 mL of n-butanol are added, vigorously shaken, and the butanol layer is separated by centrifugation. The compound from combining one molecule MDA with 2 molecules thiobarbituric acid – Knoevenagel’s reaction in the butanol layer was measured at 535 nm on a spectrophotometer and MDA concentration was calculated using a standard curve of different concentrations of 1,1,3,3-tetraethoxypropane (Sigma Aldrich Chemie GmbH, Munich, Germany).

**Catalase activity (CAT)** – was estimated using the method by Goth [17], based on the enzymatic dissociation of the substrate hydrogen peroxide (H₂O₂) and spectrophotometrical measuring of the stable yellow colored complex between H₂O₂ and ammonium molybdate at 405 nm. Briefly, 0.2 mL aqueous humor or lens homogenate sample is incubated with 1.0 mL substrate (65 mM hydrogen peroxide in 60 mM sodium–potassium phosphate buffer, pH 7.4) at 37 °C for one minute. The reaction is stopped with 1.0 mL 32.4 mM ammonium molybdate solution. Catalase concentrations were calculated against three blank test tubes that are parallelly run, namely blank 1 (containing substrate, molybdate and finally sample), blank 2 (substrate, molybdate and buffer) and blank 3 (molybdate and buffer). The absorbances of the four test tubes are measured at 405 nm. Under these conditions, one unit of catalase dissociates 1 pmol H₂O₂ for a minute.

**Ferric reducing ability of lens or aqueous humor (FRA-L or FRA-A)** – Ferric (Fe³⁺) to ferrous (Fe²⁺) ion reduction by 2,4,6-tripryridyltriazine at low pH causes a colored ferrous-tripryridyltriazine complex to form [6]. The change in absorbance is directly related to the amount of electron-donating antioxidants present in the reaction mixture. Fresh working solution is prepared by mixing 300 mmol/l acetate
buffer with pH 3.6, 10 mmol/l 2,4,6, tripyridyltriazine in 40 mmol/l hydrochloric acid and 20 mmol/l ferric chloride at a ratio of 10:1:1. Then, 1.5 ml working solution is warmed to 37 °C and its absorption is measured at 593 nm (A0) against distilled water. Then, 50 µL lens homogenate or aqueous humor sample are added and the absorption is measured again after exactly 4 minutes (A1). FRA-L (FRA-A) values are obtained by comparing the absorbance change at 593 nm (A1-A0) in test reaction mixtures against a standard curve of solutions of FeSO₄×7H₂O in the range of 100–1000 µmol/l.

Reduced glutathione (GSH) – after protein precipitation the reduced thiols interact with Ellman's reagent (5,5’-dithiobis(2-nitrobenzoic acid) - DTNB) to form a stable yellow color compound, 5-thio-2-nitrobenzoic acid (TNB) which is measured spectrophotometrically [1] at 412 nm. For this purpose, the aqueous humor or lens homogenate sample is mixed immediately with precipitating solution (1.67 g glacial metaphosphoric acid, 0.20 g Na₃EDTA, 30 g NaCl, 100 ml water), and shaken. After 5 min of incubation at room temperature the reaction mixture is centrifuged (3000×g; 15 min). To 0.5 ml of the clear supernatant, 2.0 ml of 0.3M Na₃PHO₄ phosphate solution and 250 µl DTNB solution (200 mg in 100 ml 1% sodium citrate solution) are added. A blank is prepared with 1 ml phosphate solution, 1 ml water, 0.5 ml precipitating solution, and 250 µl DTNB solution. A standard curve of reduced L-glutathione (Sigma-Aldrich, Chemie GmbH, Munich, Germany) to is used to determine the GSH concentration of samples.

Protein carbonyl groups (PCO) – they are estimated after derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl(DNP) hydrazone product. NaOH is added to the protein solution after the addition of DNPH, shifting the maximum absorbance wavelength of the derivatized protein from 370 nm to 450 nm. This reduces the interference of DNPH and allows the direct quantification in the sample solution without the need for the precipitation, washing and resuspension steps in the traditional DNPH method [29]. Samples of aqueous humor of lens homogenate (400 µL), previously diluted with phosphate buffered saline (PBS) to total protein content between 1 and 10 g/L, were incubated with 400 µL 10 mM DNPH in 0.5M phosphoric acid for 10 min. Then 200 µL 6M NaOH was added. Absorbance was read after 10 min incubation at 450 nm against a blank containing buffer instead of protein solution. The values of PCO were calculated with the help of molar absorption coefficient of hydrazine derivatives of carbonyl groups for the conditions of the reaction (22308 M⁻¹cm⁻¹).

Advanced oxidation protein products (AOPPs) – Spectrophotometric determinations of AOPP levels were performed using a method described by Hansand et al. [18]. In this assay the undiluted samples (40 µL) were mixed with 0.2M citric acid (160 µL) to dissolve the lipids and with 1.16M KI in PBS (10 µL) for quantitative measurement of oxidation of I⁻ to I³⁻. After shaking for 2 min, the absorbance of the reaction mixture was immediately read at 340 nm and was calculated in relation to standard curve of chloramine-T solutions. Calibration standards (190 µL) were mixed with 50 µL KI and their absorption was read at 340 nm. AOPP concentrations are expressed in µmol/l of chloramine-T equivalents.

Trace minerals content

Samples of healthy lens (or lens slurry) were weighed, digested with HNO₃ (65%), H₂O₂ (30%) and the digest was diluted to 2-3 mL with deionized water. The content of copper (Cu), zinc (Zn), and iron (Fe) in homogenized lenses was determined by atomic absorption spectrophotometry of previously weighed samples and liquid mineralization with 65% nitric acid and 30% hydrogen peroxide. Atomic absorption was red on an atomic absorption spectrophotometer Perkin Elmer, model Analyst 800 AA Spectrometer, USA, for work on flame, lead furnace. The calculation of trace mineral's concentrations was made with the help of calibrating straight line and wavelengths for Cu – 324.8 nm, Zn – 213.9 nm, and Fe – 248.3 nm. The results are presented in µmol/g fresh tissue.

The levels of trace minerals in aqueous humor were quantitated with commercial colorimetric kits (Fortress Diagnostics, UK).

STATISTICAL ANALYSIS

The statistical analyses were accomplished using a computer program Statistica® 68 version 7.0 (Stat Soft Inc., USA). First, a descriptive statistic of all the investigated parameters regarding mean values, standard deviations, median with minimal and maximal values, lower and upper limits of 95% confidence intervals were done. Afterward, the Kolmogorov-Smirnov test was performed to check the normality of distribution of mean data. The statistical difference between groups was then evaluated by parametric (ANOVA) or nonparametric (Mann-Whitney) tests according to data distribution pattern. The difference was accepted to be significant at p<0.05.

Results

The results showed a significant elevation of MDA, PO, AOPP, and significant reduction of GSH and Fe in aqueous humour of eyes with cataracts in comparison with non cataractous lenses (Fig. 1). MDA and Fe but not PCO and AOPP were also significantly increased in cataractous lenses while GSH and FRAL were decreased significantly compared to the healthy ones (Fig. 2).

Discussion

Almost every biological macromolecule can be oxidized by ROS, however, lipids and proteins are the most abundant and vulnerable biomolecules present in the eyeball. Our
results showed significantly higher levels of markers of lipid (MDA) and protein (PCO and AOPP) peroxidation in eyes with cataracts compared with the control healthy eyes. Chang et al. [8] and Kaur et al. [22] found out that development of cataract was connected with systemic oxidative stress based on increased serum levels of MDA in humans and dogs [27].

The increased concentration of MDA in lenses with senile cataract without systemic imbalance of the lipid redox status was reported in other investigations in humans [33]. Our results showed evidence for local lipid peroxidation in dogs with cataract as MDA levels were elevated both in lenses and aqueous humour. Madany et al. [26] also reported elevation of MDA in aqueous humour of dogs with senile cataract. It was suggested that aqueous humour micro condition correlated well with composition of lens with cataract, and can be reliable for measurement of oxidative stress parameters [20].
Lipid peroxidation is particularly destructive, for it develops as a self-perpetuating chain reaction. Proteins, peptides, and amino acids are also targets for ROS, but their modification is less harmful than those of the lipids, because the progression of reaction is slower. However, ocular tissues have a high percentage of proteins and their oxidation is important [7]. The use of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of their relatively early formation and the relative stability of carbonylated proteins. A significant amount of carbonyl groups was formed owing to glycation of the lens proteins [34]. Exposure of human lens epithelial cells to \( \text{H}_2\text{O}_2 \) significantly increased levels of oxidized proteins, lipid peroxidation product MDA, and DNA damage [12]. Kyselova et al. [25] found out higher protein carbonyl and lower protein sulphhydryl content in lenses with the advanced stage of experimental cataract when compared with the still transparent diabetic lenses in rats. In an attempt to model the processes of free radical-mediated cataractogenesis, Stefek et al. [37] investigated the oxidative modification of rat eye lens proteins by peroxyl radicals. The insolubilisation of eye lens proteins was accompanied by the accumulation of protein free carbonyls and the diminution of sulphhydryls. We also found an elevation of PCO in aqueous humor of dogs with cataract but not in lens homogenates. The same finding was observed about AOPP. Modifications of lens proteins can create a barrier between the metabolically active lens cortex and aqueous humour that prevents free diffusion of molecules between these eye media, and could explain the differences in concentrations of investigated parameters.

Advanced oxidation protein products (AOPP), a new marker of protein oxidation, have recently attracted the attention of investigators. They are formed during oxidative stress by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines (produced by myeloperoxidase in activated neutrophils). Elevated markers of protein oxidation have also been associated with cataractogenesis. Cataract human patients had higher serum levels of AOPP, and PCO with respect to the normal subjects [8]. In the present study, the level of AOPP and protein carbonyls in the eyes, were increased in aqueous humour of dogs with cataract, demonstrating the presence of protein oxidative damage in age-related cataract.

Ocular tissues are constantly exposed to sunlight, a potent source of highly reactive free radicals; however, eyes have well developed antioxidant systems that prevent damage from excessive oxygen metabolites. Diurnal animals have the highest concentrations of antioxidants - about several times higher in eye media than in plasma [24]. Reduced glutathione (GSH) is the major free thiol in most living cells and is involved in many biological processes such as detoxification of xenobiotics, removal of hydroperoxides, and maintenance of the oxidation state of protein thiolysyl.

It is the key antioxidant in animal tissues. Low levels of GSH have been observed in lenses with advanced cataract in our and other studies [24, 30, 33]. This could be the result of various events such as decreased synthesis connected to aging or increased oxidative consumption for counteraction against toxic substances.

The principal antioxidant enzymes protecting the eyes from ROS are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). Serum SOD, GSH-Px, and CAT activities in humans with age-related cataract were significantly decreased as compared to the control subjects [8]. The decreased plasma activity of antioxidant enzymes SOD and CAT, without changes in ascorbate levels in aqueous humour of poodles with and without cataract was found [5]. Catalase activity in lens epithelium cells (LECs) was significantly decreased in senile cataract as compared to normal healthy control cases, but there was no significant difference of catalase activity in all morphological types of cataract [14]. In the same study total antioxidant capacity (TAC) in LECs was significantly decreased in senile cataract and there was significant difference of TAC for different morphological types of senile cataract.

In our study we found out a significantly decreased GSH levels both in aqueous humour and lenses of eyes with cataract comparatively to healthy eyes but no significant difference in CAT activities. Total antioxidant capacity of the aqueous humour was not changed, whereas ferric-reducing ability of cataract lenses (FRAL) was significantly less in comparison with healthy dogs. The serum total antioxidant status was reported to be diminished in dogs with age-related cataract [27]. The levels of both the plasma and the lens ferric reducing ability were significantly decreased in the age-related and diabetic cataracts as compared to those in the controls [21]. Ksic et al. [24] found out a higher ferric-reducing ability in lenses of patients with insipient compared to mature age-related cataract. Similarly, our results indicate that decreased FRAL in cataractous lenses compared to healthy ones corresponded to the lowest levels of GSH. Therefore, with progression of cataract maturity the total antioxidant ability of lenses decreases as a result of consumption and depletion of antioxidants.

There is a close association between minerals and antioxidant defense of the eye [11]. Atomic absorption spectrophotometry is a highly sensitive method that can detect metals in concentrations as low as 1 ppm. We used this method to measure the content of the most important trace minerals in eye media with cataract. The amounts of Cu and Zn in both lenses and aqueous humour did not differ significantly between cataractous and healthy eyes, just opposite to Fe which was significantly decreased in aqueous and increased in lenses with cataract comparatively to eyes of non-affected dogs. Scientific reports regarding mineral levels in the eyes with cataract are contradictory. Zinc and Cu contents of human lenses were elevated in mature cataract
Iron content has been reported to be increased in lenses with senile cataract [9]. According to Goralska et al. [16] low oxygen level stimulates the lens epithelial cells to trap the cytosolic iron and increases the risk for iron-induced formation of ROS in the lens. Iron concentration is tightly connected to its transport proteins transferrin and ferritin; their levels have been also changed in intraocular tissues during cataract and other pathological conditions [13]. Prooxidative properties of free iron and copper are due to their interaction with \( \text{H}_2\text{O}_2 \), that results in formation of the most reactive oxygen species: the hydroxyl radical through Fenton reaction [38]. The level of free iron is tightly regulated and kept at a low level by binding with ferritin, which is responsible for sequestering iron in the cytoplasm and nucleus in non-toxic form [28]. Dog lens epithelial cells in culture were able to synthesize and secrete ceruloplasmin. Under physiological conditions, ceruloplasmin increased iron incorporation into the storage protein, ferritin. Under conditions of iron overload, it decreased iron incorporation into ferritin, which is consistent with increased efflux of iron. These effects indicate an important role for these proteins in iron metabolism in the lens [19].

In conclusion, our study with dogs in a clinical setting confirmed the evidence from experimental and human investigations that lipid and protein peroxidation occurs in eyes with senile cataract. Moreover, local antioxidant defense seemed to be insufficient to overcome excessive ROS production and resulting oxidative stress.

**Conflict of Interest**

The authors declare no conflicts of interest.

**References**


